

High Efficiency *In vitro* Regeneration and Genetic Stability of *Corallocarpus epigaeus* - An Endangered Medicinal Plant

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Abstract

Indirect regeneration of plantlets from multiple shoot induction of *Corallocarpus epigaeus* was obtained from leaf and nodal explants on MS with different concentrations of BAP in combination with IAA/IBA or IBA alone. Among all the combinations, BAP and IBA exhibited maximum regeneration. High frequency of multiple shoots (89%) was obtained on BAP (2.0 mg/l) and IBA (1.5 mg/l) in nodal explants. Maximum mean shoot length of 6.8 ± 0.33 cm was obtained in nodal explants cultured on BAP (1.0 mg/l) + IBA (0.5 mg/l), followed by leaf explants with 6.7 ± 0.47 cm on BAP (3.0 mg/l) + IAA (2.5 mg/l). The highest frequency of rooting (88.3%) was obtained on NAA (1.0 mg/l) and IBA (2.0 mg/l) with 21.83 ± 0.57 mean number of roots. The well-rooted healthy plantlets were acclimatized with a survival rate of 80%. Inter simple sequence repeat (ISSR) analysis revealed the genetic similarity of *in vitro* raised plants with the mother plant.

Introduction

Corallocarpus epigaeus, a medicinal endangered tuberous climber belongs to Cucurbitaceae. It is a monoecious climber. This species is globally distributed in tropical Africa, and southeast Asia. In India it has been recorded from Punjab, Madhya Pradesh, West Bengal, Bihar, Uttar Pradesh, Telangana and Andhra Pradesh (Umadevi and Kamalam 2012). *C. epigaeus* is commonly called as Akas Gaddah. *C. epigaeus* plant is one such important medicinal plants used in treating syphilis, venereal complaints and chronic dysentery (Kothawade and Siddiqui 2018); it is also effectively used to cure diabetes (Gnananath et al. 2013), snakebites (Chandrakala et al. 2013) and rheumatism (Uthrapathy et al. 2011).

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It also possesses anthelmintic property (Kirubha et al. 2011), anti-fertility (Dhanapal et al. 2006), haematopoietic (Saranya et al. 2017), hepatoprotective (Mahesh et al. 2012), antioxidant and anti-inflammatory (Jeyaseelan et al. 2014) activities.

The medicinal value of plants contains some chemical substances, usually secondary metabolites, which produce a definite physiological action on the human body. As per the above reason, it is very essential for conservation and *in vitro* propagation. The *in vitro* propagation studies were carried out in *C. epigaeus* for protection of species with the help of leaf and node explants, due to lack of a suitable method for natural regeneration and over-exploitation of *C. epigaeus*, which severely deteriorated the species consequently recording them as endangered species (Vemula et al. 2020). Due to exploitative harvesting of tubers for trade and denudation of forests, the natural population of *C. epigaeus* has declined to such an extent that it is now considered rare and threatened in its natural habitats (Narayan 2016).

Screening of genetic fidelity is also important to study the genetic variations in *in vitro* regenerated plants with the parents. The somaclonal variations may occur due to explant type, phytohormones, and subsequent subculturing during micropropagation (Lakshmanan et al. 2007). ISSR molecular markers are used to screen total plant genome coding and non-coding sequences and discover the genetic variations (Collard and Mackill 2009). The present study reveals the high frequency of *in vitro* regeneration and genetic fidelity analysis of regenerated plants of *C. epigaeus*. Further, it is necessary to design *in vitro* acclimatized plantlet roots by treating with bio-inoculates (PGPR, *Azotobacter*, PSM and *Mycorrhiza*) for evaluating their survival rate in abiotic stress conditions.

Materials and Methods

The tuberous root of *Corallocarpus epigaeus* was collected from Asifabad Forest, Telangana State, India during monsoon season. The plants were grown and maintained at departmental greenhouse, Department of Biotechnology, Kakatiya University, Telangana State, India. Young leaves and nodes were collected, washed thoroughly and subsequently surfaced sterilized with 2% (w/v) Tween-20 (for microbial disinfection) for 3 - 4 min, then rinsed with distilled water for 3 - 4 times. The explants were transferred to laminar airflow for further sterilization. The explants (leaf and node segments) were sterilized with 0.1% (w/v) HgCl_2 for 2 - 3 min and then rinsed again with sterilized distilled water to remove any traces of HgCl_2 .

MS provided with sucrose (3%) and myo-inositol (100 mg/l) was used throughout the study. The pH was adjusted to 5.7 with 0.1N NaOH or 0.1N HCl and addition of agar (0.8%) resulted in the solidification of the medium. The medium was sterilized at 121°C for 15 min under 15 psi pressure. The surface sterilized leaf and nodal explants were inoculated and maintained at $25 \pm 2^\circ\text{C}$ under a 16/8 hrs light and dark cycle provided by cool fluorescent tubes.

The explants cultured on MS with different concentrations of BAP in combination with IAA/IBA or IBA alone induced callus and upon subculturing induced multiple shoots from callus. Regularly, the cultures were subcultured onto the fresh medium for 4-5 weeks intervals. The observations were made every six days of inoculation. All the experiments were conducted thrice with 60 explants for each treatment. The rate of multiplication represents the number of shoots produced per explant on a specific medium after the number of days of its inoculation as mentioned in the results. Data were collected and analyzed after 45 days.

The individual *in vitro* raised multiple shoots developed from calli were excised and subcultured onto half-strength MS including plant growth regulators IBA/NAA/IAA alone (at a concentration of 0.2 - 1.5 mg/l) for adventitious roots. The data on the number of roots and root length were recorded after 3 weeks. The significant differences with different parameters recorded were calculated by mean and standard error variance (ANOVA) using SPSS software.

After 3 weeks of root initiation, plantlets were collected and rinsed with sterile distilled water to remove the traces of nutrient medium. Plantlets were transplanted into the pots containing sterile black soil, peat, and sand (2 : 1 : 1) (Fig. 2e,f). The pots were covered with transparent polythene covers to maintain the growth conditions and humidity of about 70 - 80%, light 60 $\mu\text{mol}/\text{m}^2/\text{s}$, at $25 \pm 2^\circ\text{C}$. These transplanted plants were kept in the culture room and irrigated every alternate day with sterile water for three weeks. After three weeks of hardening, the plantlets were exposed to the field and transferred to greenhouse for further acclimatization.

Assessment of genetic fidelity of *C. epigaeus* plants was made through ISSR analysis. Genomic DNA was isolated from the mother plant and regenerated plantlets by C-TAB method (Doyle and Doyle 1987) and amplified by PCR using ISSR primers. Three ISSR primers (Hy2: AGAGAGAGAGAGAGAGG, Hy3: TCTCTCTCTCTCTCG, and Hy5: AGAGAGAGAGAGAGAGYT) were chosen from 8 tested primers based on their compatibility (Table 3). Using these three primers, mother plant and *in vitro* regenerated plants' DNA were amplified in PCR, conditions following (initial denaturation 94°C for 5', 30 cycles of 94°C 30 Sec, 50°C 30 Sec, 72°C for 1 min followed by final extension of 72°C for 7') and chemicals used for the PCR reaction were from Fermentas, Maryland, USA. Amplified PCR products were examined by agarose gel electrophoresis (1%) and photographed using Gel Doc XR+ (Bio-Rad).

Results and Discussion

The explants cultured on basal MS without any plant growth regulators (PGRs) had no morphogenetic response of either callus or multiple shoot induction. The leaf and nodal explants cultured onto MS with varying concentrations of BAP + IAA, BAP + IBA and IBA alone induced callus from the cut ends of the explants. Excessive green callus was obtained on MS augmented with IBA (1.0 mg/l) from both leaf and nodal explants (Figs

1b, 2b), whereas in the previous studies reported in the same plant, quick callus growth was obtained on MS with BA (2.0 mg/l) and NAA (0.5 mg/l) from stem and leaf explants (Narayan 2016). Similar results of green callusing on MS with IBA was obtained in *Gossypium hirsutum*, where IBA (1.5 mg/l) achieved 86.6% of green callus (Abdellatef and Khalafallah 2008) and in *Jatropha curcas* IBA induced green callus which later turned brown (Rajore and Batra 2007). But in contrast, a high percentage of callogenesis and excessive green callusing was achieved in *Citrullus colocynthis* on Kn and TDZ supplemented medium (Dasari et al. 2015).



Fig.1. Effect of PGRs on indirect plant regeneration from leaf explants of *Corallocarpus epigaeus*. a. Initiation of callus on 1.0 mg/l IBA. b. Profusely induction of light green callus on MS with 1.0 mg/l IBA. c. Initiation of micro-shoots at 1.5 mg/l IAA and 2.0 mg/l BAP. d. Formation of multiple shoots and elongation on 1.5 mg/l IBA and 2.0 mg/l BAP (rooting was shown in insight). e. High frequency of multiple shoots with roots was formed on MS with 1.5 mg/l IAA and 2.0 mg/l BAP.

The same callus was subcultured on half-strength MS with the same plant growth regulators. Among all the combinations and concentrations, IBA + BAP was found to be efficient. High regeneration frequency of multiple shoots (89.33%) was obtained in nodal explants, followed by leaf explants (86.00%) on MS with IBA (1.5 mg/l) and BAP (2.0

mg/l). The optimum mean number of shoots (15.7 ± 0.81 and 13.8 ± 0.95) was obtained in nodal and leaf explants on the same medium (Figs 1d, 2c and Table 1). The maximum mean shoot length of 6.8 ± 0.39 cm was obtained on IBA (0.5 mg/l) and BAP (1.0 mg/l) in nodal explants (Fig. 2d), whereas the maximum shoot length of 6.7 ± 0.47 cm was obtained on MS with IAA (2.5 mg/l) and BAP (3.0 mg/l) in leaf explants (Fig. 1e and Table 1).

Table 1. Effect of PGR's on node and leaf explants of *Corallocarpus epigaeus* in the induction of multiple shoots.

PGR's conc. (mg/l)	Regeneration		Mean no. of shoots \pm SE		Mean shoot length (cm) \pm SE		
	Node	Leaf	Node	Leaf	Node	Leaf	
IBA							
0.5	68.33 ± 0.39	64.23 ± 0.29	6.3 ± 0.6	5.9 ± 0.3	5.5 ± 0.47	5.3 ± 0.34	
1.0	75.67 ± 0.65	73.37 ± 0.97	7.2 ± 0.5	7.1 ± 0.5	5.1 ± 0.43	4.7 ± 0.40	
1.5	77.37 ± 0.14	76.33 ± 0.51	13.1 ± 0.8	12.2 ± 0.6	3.3 ± 0.28	3.2 ± 0.36	
2.0	78.0 ± 0.25	79.13 ± 0.68	12.4 ± 0.9	11.3 ± 0.4	4.2 ± 0.39	4.1 ± 0.45	
2.5	66.67 ± 0.53	70.33 ± 0.14	8.5 ± 0.38	9.4 ± 0.51	4.7 ± 0.20	4.3 ± 0.37	
IAA + BAP							
0.5	1.0	67.33 ± 0.74	66.67 ± 0.37	5.2 ± 0.61	5.1 ± 0.71	6.5 ± 0.63	6.4 ± 0.37
1.0	1.5	74.67 ± 0.90	73.33 ± 0.83	8.3 ± 0.20	7.9 ± 0.29	4.3 ± 0.37	5.5 ± 0.25
1.5	2.0	85.67 ± 0.14	84.0 ± 0.25	12.5 ± 0.87	11.4 ± 0.8	3.4 ± 0.56	3.6 ± 0.35
2.0	2.5	75.33 ± 0.23	76.27 ± 0.53	9.2 ± 0.41	9.4 ± 0.32	4.1 ± 0.29	4.5 ± 0.40
2.5	3.0	65.0 ± 0.1	65.78 ± 0.75	4.3 ± 0.72	4.8 ± 0.91	5.4 ± 0.42	6.7 ± 0.47
IBA + BAP							
0.5	1.0	67.37 ± 0.53	66.37 ± 0.90	6.3 ± 0.37	6.2 ± 0.39	6.8 ± 0.39	5.8 ± 0.57
1.0	1.5	76.67 ± 0.14	75.27 ± 0.65	7.8 ± 0.31	9.6 ± 0.29	5.3 ± 0.40	4.9 ± 0.63
1.5	2.0	89.33 ± 0.39	86.0 ± 0.78	15.7 ± 0.81	13.8 ± 0.9	3.2 ± 0.54	3.4 ± 0.45
2.0	2.5	76.43 ± 0.97	73.0 ± 0.51	11.7 ± 0.63	7.9 ± 0.38	4.0 ± 0.47	5.4 ± 0.29
2.5	3.0	65.0 ± 0.77	67.32 ± 0.29	8.7 ± 0.44	6.9 ± 0.49	5.9 ± 0.35	6.2 ± 0.44

However, the regeneration studies carried out previously in the same plant showed the maximum percentage response of 68.33 on BA (0.5 mg/l) + IAA (2.0 mg/l) (Narayan 2016) and 88 on TDZ (1.5 mg/l) + IAA (1.5 mg/l) (Vemula et al. 2020). The combination of IBA and BAP was also found to be effective in species like *Bryonopsis lacinosa*, where IBA and BAP induced multiple shoots from cotyledon derived callus (Caroline and Mallaiah 2011), whereas in *Cicer arietinum*, IBA and BAP resulted in maximum percentage of response in all explants (Sadhu et al. 2020). But in contrast, only BAP alone induced

multiple shoot regeneration in several plants like *Cucurbita pepo* and *Beninca sahisvida* (Haque et al. 2008), *Momordica dioica* (Devendra et al. 2009), lentils (Ozdemir and Turker 2014), *Ficus* (Hesami et al. 2018) and *Cucumis sativa* (Venkatachalam et al. 2018).



Fig. 2. *In vitro* regeneration of *Corallocaepus epigaeus* from nodal explants. a. Initiation of callus from mature nodal explants. b. Induction of complete light green callus from nodal explants. c. Initiation and proliferation of multiple shoots from light greenish callus. d. Elongated multiple shoots transferred to rooting medium (rooting was shown in insight). e. *In vitro* regenerated plantlets. f. Partially hardened plants.

The combination of BAP and IAA showed less regeneration in *C. epigaeus* when compared to other combinations. Present results do not agree with the reports of other plants, where BAP and IAA induced adventitious shoots in *Beninca sahisvida* (He et al. 2006) and high concentration of BAP and IAA induced shoot regeneration in *Citrullus vulgaris* (Dong and Jia 1991). BAP and IAA also induced multiple shoot regeneration in *Anisochilus cornosus* (Reshi et al. 2017), *Bambusa glaucescens* (Shirin and Rana 2007), and *Lagenaria siceraria* (Saha et al. 2007).

The highest frequency of rooting (88.3 ± 0.14) was obtained on MS with NAA (1.0 mg/l) and IBA (2.0 mg/l) with 21.83 ± 0.57 mean number of roots from nodal explants and

19.36 ± 0.22 from leaf explants. The highest mean root length of 5.6 ± 0.62 cm was obtained on NAA (0.5 mg/l) and IBA (1.0 mg/l) from leaf explants and 5.2 ± 0.45 cm on IBA (1.5 mg/l) in nodal explants (Table 2). Similar results were obtained in *Citrullus colocynthis*, where NAA and IBA, IAA and IBA showed the best rooting (Krishna and Shastree 2015). Kumar et al. (2003) also reported similar results in *Cucumis sativa* and NAA and IBA proved to be best for callus induction as well as rooting from leaf and stem explants of *Heliotropium indicum* (Bagadekar and Jayaraj 2011).

Table 2. Effect of various PGRs in induction of roots of *Corallocarpus epigaeus*.

PGR's conc. (mg/l)	% of culture producing roots		Mean no. of roots ± SE		Mean length of roots (cm) ± SE	
	Node	Leaf	Node	Leaf	Node	Leaf
IBA						
0.2	68.3 ± 0.83	77.0 ± 0.51	5.67 ± 0.74	5.36 ± 0.42	3.6 ± 0.33	3.4 ± 0.46
0.5	75.6 ± 0.74	84.3 ± 0.78	6.12 ± 0.51	7.22 ± 0.31	2.8 ± 0.41	1.8 ± 0.32
1.0	86.0 ± 0.51	65.6 ± 0.39	7.44 ± 0.39	4.96 ± 0.34	1.9 ± 0.44	3.9 ± 0.49
1.5	66.0 ± 0.68	55.0 ± 0.25	4.92 ± 0.48	3.49 ± 0.45	5.2 ± 0.45	4.9 ± 0.61
NAA						
0.2	67.0 ± 0.25	75.0 ± 0.25	4.92 ± 0.19	5.41 ± 0.75	3.8 ± 0.25	3.2 ± 0.49
0.5	76.3 ± 0.29	80.6 ± 0.39	6.51 ± 0.47	6.49 ± 0.52	2.5 ± 0.66	2.6 ± 0.88
1.0	82.0 ± 0.77	63.6 ± 0.65	7.0 ± 0.25	5.63 ± 0.34	1.8 ± 0.53	3.1 ± 0.75
1.5	74.6 ± 0.74	51.6 ± 0.29	5.43 ± 0.55	4.54 ± 0.48	2.7 ± 0.23	4.4 ± 0.66
NAA+IBA						
0.5 + 1.0	65.4 ± 0.62	63.4 ± 0.32	13.46 ± 0.86	11.32 ± 0.36	5.1 ± 0.82	5.6 ± 0.62
0.5 + 2.0	68.6 ± 0.25	66.2 ± 0.74	15.91 ± 0.92	14.21 ± 0.29	4.2 ± 0.51	4.5 ± 0.76
1.0 + 1.0	77.3 ± 0.90	75.1 ± 0.83	18.55 ± 0.66	16.29 ± 0.44	4.7 ± 0.56	3.9 ± 0.69
1.0 + 2.0	88.3 ± 0.14	87.0 ± 0.35	21.83 ± 0.57	19.36 ± 0.22	3.3 ± 0.44	3.5 ± 0.40

ISSR markers were used to assess the genetic fidelity between the mother plant and the regenerated plants of *C. epigaeus* plants. A similar kind of genetic fidelity evaluation was successfully obtained in different plant systems like *Citrullus colocynthis* (Dasari et al. 2015), *Mentha arvensis* (Faisal et al. 2014), *Corallocarpus epigaeus* (Vemula et al. 2020), *Momordica cymbalaria* (Chaitanya et al. 2020), *Ocimum tenuiflorum* (Aggarwal et al. 2020), *Cicer arietinum* (Sadhu et al. 2020) and *Sapium sebiferum* (Hou et al. 2020) using ISSR assessment. The ISSR analysis confirmed that there is no polymorphic nature between regenerated and mother plants, both plants exhibited similar monomorphic banding patterns ranged between 100 bp and 2.5 kb. Therefore, the results showed that the mother plant and regenerated plant contain the same gene pool (Fig. 3 and Table 3). ISSR analysis revealed the genetic stability of *in vitro* raised plants with the mother plant.

The present study demonstrated an efficient regeneration protocol through indirect organogenesis using leaf and node explants of *Corallocarpus epigaeus* and assessment of genetic fidelity demonstrates the true-to-type nature of regenerated plants comparatively to the mother plant. The combination of BAP (2.0 mg/l) and IBA (1.5 mg/l) were shown to be best for maximum regeneration of 89.33% with 15.7 ± 0.81 mean number of shoots in nodal explants. The highest mean shoot of 6.8 ± 0.39 cm was obtained at IBA (0.5 mg/l)

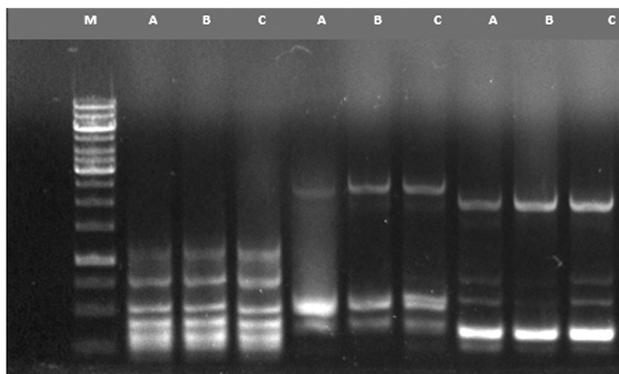


Fig. 3. Genetic fidelity of regenerated plantlets. M = Marker, A = Mother plant, B-C = Regenerated plantlets.

Table 3. Genetic fidelity of *in vitro* regenerated plantlets using 3 different primers.

Sl. no.	Primers	Primer sequence	Size of the band
1	Hy2	AGAGAGAGAGAGAGAGG	100 - 1 kb
2	Hy3	TCTCTCTCTCTCTCG	500 - 2.5 kb
3	Hy5	AGAGAGAGAGAGAGAGYT	100 - 2.0 kb

and BAP (1.0 mg/l) in nodal explants and 6.7 ± 0.47 in leaf explants on MS with IAA (2.5 mg/l) and BAP (3.0 mg/l). The induced shoots transferred to half-strength MS with NAA/ IBA/ NAA and IBA for rooting, better results were shown at 1.0 mg/l NAA and 2.0 mg/l IBA with a maximum mean number of roots of 21.83 ± 0.57 in nodal explants. Further *in vitro* regenerated acclimatized plantlets are using for isolating and screening of secondary metabolites from various plant parts. For the above reasons, biotechnological analysis studies are required in *C. epigaeus*.

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